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Review

Pleiotropic mechanisms of action of perhexiline in heart failure

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Abstract

Introduction: The re-purposing of the anti-anginal drug perhexiline (PHX) has resulted in symptomatic improvements in heart failure (HF) patients. The inhibition of carnitine palmitoyltransferase-1 (CPT-1) has been proposed as the primary mechanism underlying the therapeutic benefit of PHX. This hypothesis is contentious.

Areas covered: We reviewed the primary literature and patent landscape of PHX from its initial development in the 1960s through to its emergence as a drug beneficial for HF. We focused on its physico-chemistry, molecular targets, tissue accumulation and clinical dosing.

Expert opinion: Dogma that the beneficial effects of PHX are due primarily to potent myocardial CPT-1 inhibition is not supported by the literature and all available evidence point to it being extremely unlikely that the *major* effects of PHX occur via this mechanism. *In vivo* PHX is much more likely to be an inhibitor of surface membrane ion channels and also to have effects on other components of cellular metabolism and reactive oxygen species (ROS) generation across the cardiovascular system. However, the possibility that *minor* effects of PHX on CPT-1 underpin disproportionately large effects on myocardial function cannot be entirely excluded, especially given the massive accumulation of the drug in heart tissue.

Keywords: perhexiline, carnitine palmitoyltransferase, heart failure, metabolism, modulation, therapy

Article highlights

- In contrast to recent dogma, we propose that it is unlikely that the therapeutic benefit of perhexiline in patients with HF is due, in large part, to the inhibition of CPT-1.
- All available data suggests that PHX is a drug of low potency, low specificity and low selectivity that interacts with multiple surface membrane ion channels and other intracellular components that impinge on cellular metabolism and the generation of reactive oxygen species (ROS).
- The possibility that *minor* effects of PHX on CPT-1 underpin disproportionately large effects on myocardial function cannot be excluded, especially given the massive accumulation of PHX in myocardial tissue.
- A scheme is described that considers the pleiotropic effects of PHX via the inhibition of surface membrane ion channels and its accumulation in mitochondrial membranes.
- The combined action of PHX on multiple targets throughout the cardiovascular system probably underpins the symptomatic improvements in HF patients.

(1) Introduction- new therapies for heart failure (HF).

As defined by Yancy and colleagues "HF is a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood".[1] It is a global epidemic, affecting 10% of individuals over the age of 65 years and its clinical progression results in mortality of 50% within 4 years of diagnosis.[1] Contemporary approaches, which aim to correct left ventricular ejection fraction (LVEF) and halt or normalize the progression of myocardial dysfunction are sub-optimal and involve the modulation of neurohumoral targets including β-adrenergic (β-AR) blockers, suppression of the renin-angiotensin-aldosterone (RAA) system (angiotensin II receptor (ATIIR), angiotensin-converting enzyme (ACE), aldosterone antagonism), vasodilators and diuretics. Whilst these conventional therapies primarily modulate upstream regulators of the circulatory system, there are newer strategies that aim to target more 'myocardially-focused' elements including the drivers of progressive contractile and arrhythmogenic events that occur *within* cardiac cells[2-5] and even approaches that replace or repair damaged myocardium.[6]

We have previously described normal myocardial cell signaling in terms of the synchronization of coupled systems.[7-9] For example, there is functional integration between cellular ion fluxes and metabolism in which the dynamic cycling of intracellular Ca^{2+} is critically modulated by ATP-dependent processes, and many events in ATP synthesis are Ca^{2+}-dependent. Consequently, the functional deterioration of the myocardium in long-term cardiac diseases such as HF is associated with the progressive desynchronization within and between numerous linked processes. Using the example above, disease-linked derangement in Ca^{2+} signaling, which is considered a major feature of HF pathogenesis, impacts on ATP bioavailability and vice versa.[7, 8]

If we consider then that approaches that aim to correct HF-linked Ca^{2+} signaling dysfunction[7, 8, 10-14] may positively influence other 'linked' systems such as metabolism, there is substantial merit in developing new therapies that alter cellular metabolism with a view to rescuing cellular Ca^{2+} handling. An emergent approach to normalizing cardiac performance in HF therefore is the direct modulation of the myocardial metabolic state and drugs that

promote a switch in substrate utilization from fatty acid oxidation (FAO) to glycolysis (so-called 'metabolic modulators') are keenly sought.[15-18]

Important problems in developing new therapeutic approaches though include the limitations of rational drug design and sometimes prohibitory regulatory and financial barriers which have led to creative solutions for finding new niches for existing drugs in the clinical landscape.[4, 19-23] 'Repositioning', 'repurposing' and 'reappropriating' have become buzzwords in the contemporary drug development framework.

In this article, we consider some of these issues relating to perhexiline (PHX; 2-(2,2-dicyclohexylethyl)piperidine), a drug reappropriated from the treatment of angina pectoris (AP) and reported to be a clinically useful drug in the management of HF. The history of PHX, from its initial development as an L-type Ca^{2+} channel blocker[24-29], its efficacy in AP[29-35], its contraindications, withdrawals and now its emergence in treating HF has been comprehensively reviewed previously.[15, 18, 36] However, its mechanism of action remains contentious and here we consider, with specific reference to the patent landscape, how the apparent disconnect between the measureable clinical benefits of PHX in HF and the molecular underpinnings of its actions can be reconciled.

(*2) Promoting the shift from FAO to glycolysis in HF – the rationale for carnitine palmitoyl transferase (CPT) inhibition.*

The hallmark metabolic dysfunction in HF is the intrinsic shift from FAO (aerobic) to glucose (anaerobic) metabolism.[37-42] This transition is likely the consequence of a lack of adequate cardiac tissue oxygenation in the diseased state- although this is questionable in non-ischaemic HF (DCM)[43]- but it may, to some extent, represent an adaptatory mechanism to preserve myocardial function. Although glucose metabolism yields much less ATP than FAO (a theoretical yield of 38 moles ATP for the complete oxidation of glucose versus 129 moles ATP per mole of palmitate)[44], it is a more oxygen efficient mode of energy production yielding 3.17 moles ATP/moles atomic oxygen versus 2.8 moles of ATP/moles atomic oxygen for FAO of palmitate.[44] Approaches that aim to promote this 'oxygen sparing' effect

would also be coupled to the reduced flux of intermediates through the tricarboxylic acid (TCA or Krebs cycle) which would decrease the generation of reactive oxygen species (ROS) and minimize lactate production thereby preventing acidosis (Figure 1). Potentially, the combined effects of reduced ROS and normalized pH may contribute more to the beneficial effect of 'metabolic modulation' than considerations regarding the lower net yield of ATP. Since metabolism is enmeshed with numerous other cellular process (e.g. mTORC1/AMPK-mediated nutrient sensing modules involved in cell growth and autophagy)[45-48], there are other considerations for 'metabolic modulation' beyond ATP synthesis and consumption and energy substrate utilization.

Singh and colleagues have recently reviewed candidates for therapeutic metabolic modulation including the TCA cycle, pyruvate dehydrogenase (PDH), malonyl CoA-decarboxylase (MCD), fatty acid oxidation (FAO), insulin sensitivity and carnitine palmitoyltransferase (CPT-1).[18] Of these, it is CPT-1, an outer-mitochondrial membrane-resident enzyme, that has received the most attention. There are three isoforms of CPT-1 (1A, 1B and 1C); CPT1A and B share 63% sequence homology and are abundantly expressed in a wide variety of tissues, whereas CPT-1C is restricted to the brain.[49] CPT-1B is the predominant isoform in the heart and exhibits a lower affinity for its substrate carnitine than CPT-1A (approximately 15-fold) but is more sensitive to inhibition by the endogenous inhibitor malonyl co-A inhibition than CPT1A.[49] CPT-1 catalyzes the formation of acyl carnitine, via the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to carnitine[50]. Acyl carnitine subsequently transfers from the cytosol into the mitochondrial inter-membrane space to undergo CPT-2-catalyzed conversion back to acyl-CoA which undergoes catabolic beta-oxidation in the mitochondrial matrix to yield acetyl-CoA which then enters the TCA cycle (Figure 1). In principle therefore, CPT-1 inhibition, which *in situ* is mediated by of malonyl Co-A levels (Figure 1), reduces mitochondrial import and oxidation of fatty acids, promotes glycolysis via reduced acetyl-CoA production and decreases ROS and lactate accumulation (Figure 1) albeit at the expense of ATP production.

One approach to therapeutically target CPT-1 activity is the development of heterocyclic and piperidine compounds that inhibit malonyl-CoA decarboxylase (MCD) which leads to an accumulation of malonyl-CoA[51, 52], the endogenous inhibitor of CPT-1 *in vivo* (Figure 1).[53] We return to the subject of malonyl-CoA inhibition of CPT-1, and the implications for adjunctive PHX-mediated inhibition, in section 4.

However, in the context of treating chronic myocardial dysfunction, it is the direct pharmacologic inhibition of CPT-1 that has captured the imagination. In a compelling series of clinical studies, PHX (hailed as the 'forerunner of metabolic agents'[16]) has been shown to produce beneficial effects in patients with refractory HF symptoms despite otherwise optimal medical therapy [54], symptomatic hypertrophic cardiomyopathy (HCM)[55], and non-ischaemic dilated cardiomyopathy (DCM).[56] Such symptomatic improvements include reduced New York Heart Association (NYHA) classification of disease severity [54, 55], increased peak oxygen uptake (VO_2 max) [54, 55], improvements in quality-of-life scores[54, 56, 57] and six-minute walk tests[57]. Whether these functional benefits are dependent on improved myocardial function (e.g. left ventricular ejection fraction; see [54] vs. [56]), or interestingly skeletal muscle function[54], has yet to be fully established. Moreover, although these investigations evidence beneficial changes in the phosphocreatine/ATP ratio which suggest generalized improvements in cardiac energetics, in their study of DCM patients Beadle *et al.* failed to find evidence of altered cardiac substrate utilization[56]. This finding is at odds with the principal dogma of earlier studies on PHX that its main effect is to promote the shift from FAO to glycolysis.[25, 29, 58, 59] It is necessary therefore to harmonize the therapeutic benefit of PHX with its mechanism of action and in sections 3 to 6 we address this issue by reference to the patent literature and the physico-chemical determinants of CPT-1 inhibition.

(3) PHX, CPT-1 and the patent landscape.

Notwithstanding some of the disparate effects of PHX in patients with different forms of chronic heart disease[54-56], an important question is to what extent does CPT-1 inhibition contribute to the clinical improvement measured in these patients. The first patent relating to

PHX and CPT-1 was filed by Horowitz and Kennedy[60] and sought to protect the use of a screening assay for discriminating compounds on the basis of CPT-1 inhibition. Such compounds were proposed to be useful in treating 'ischaemic conditions'.

On the basis of demonstrating improved clinical endpoints in PHX-treated patients, Frenneaux and colleagues have filed a suite of patents pertaining to its efficacy in ischaemic and non-ischaemic HF[61], HF with preserved ejection fraction (HFpEF)[62] and hypertrophic cardiomyopathy (HCM).[63] These patents all include the phrase "Perhexiline (2-(2,2-dicyclohexylethyl)piperidine) is a known anti-anginal agent that operates principally by virtue of its ability to shift metabolism in the heart from free fatty acid metabolism to glucose, which is more energy efficient", but it is striking that none of these patents claim that the clinical benefit of PHX is because of CPT-1 inhibition.

However, the CPT-1-centricity of the proposed mechanism of PHX action persists and a recent patent filing from the University of Aberdeen seeks to protect intellectual property (IP) around a fluorinated PHX-derivative useful for treating a very broad range of "disorders that are ameliorated by the inhibition of carnitine palmitoyltransferase……".[64]

With regard to the progression of HF being a consequence of the deterioration of linked systems (see section 1), it is interesting to note that the patent on PHX effect in ischaemic and non-ischaemic HF[61] states that "metabolic manipulation with PHX is effective in modifying not an inciting influence, but rather the common programme of the chronic heart failure". It is plausible therefore that the metabolic dysfunction in HF is secondary to the abnormality in some other linked system and that PHX may be useful in modifying the chronic metabolic remodelling associated with HF progression and not in preventing disease onset.

(4) Is PHX a physiologically relevant CPT-1 inhibitor?

The symptomatic improvement in HF following PHX administration has been linked to a broad range of phenomenological descriptions including cGMP-dependent potentiation of platelet sensitivity to NO[32, 35, 65], vasodilatory effects[66, 67], positive inotropic actions via

troponin-C[68], circulating levels of ROS[69] and insulin sensitization.[70] However, assigning the molecular target(s) of PHX that underlies these phenomena has proved more difficult.

The patent filing of Horowitz and Kennedy[60], together with the companion paper from the same group[71], used an *in vitro* (intact) mitochondrial assay to robustly make the case that PHX (and also amiodarone) was a CPT-1 inhibitor. It was here that the concept that the "major biochemical basis of the anti-ischaemic effect of PHX is inhibition of CPT-1" was born. Measuring drug IC_{50} values in this type of assay is prone to influence from several variables, including the levels of the protein in the tissue, local concentration of drug and the accessibility of its binding site (i.e. isolated mitochondrial preparations). To our knowledge, the binding affinity of PHX for CPT-1 has not been determined. We acknowledge that it is difficult to experimentally mimic *in vitro* the precise conditions that may be important determinants of drug-target interaction *in vivo*. Although we consider investigations using recombinant CPT isoforms expressed in methylotrophic *Pichia pastoris* yeast to constitute the most direct measurements of PHX-CPT interaction[72, 73], the likelihood of PHX binding multiple targets (1) makes it impossible at present to assign the relative contributions of PHX modulation of individual targets to the downstream effects. As has been noted previously, these issues relating to 'holistic' assessments of drug action are presently beyond the resolution of contemporary assays.[22]

On the basis of Kennedy's early work on CPT1 inhibition by PHX[71], the review literature on PHX perpetuates the idea that the primary mechanism of action the drug is through CPT-1 inhibition (for example, "the identification of CPT-1 as perhexiline's major biochemical site of inhibition"[15]). Given the paucity of supporting data, the evidence base for this assertion is not convincing (Table 1).

PHX shares common properties with other Na^+-, K^+- and Ca^{2+}- ion channel blockers, many of which are recognized anti-arrhythmic drugs (Table 2), including its appreciable hydrophobicity and an ionizable nitrogen which is positively charged at neutral pH (7.4). In this regard, PHX is chemically different to etomoxir, which is an exemplar CPT inhibitor[49] and in a quasi-exhaustive review of CPT inhibitor chemistry, PHX, oxfenicine (where the

active metabolite is 4-hydroxyphenylglyoxylate (HPG)) and amiodarone were clearly categorized as “miscellaneous compound[s] reported to be CPT inhibitors”.[49] Ceccarelli *et al.* concluded that in view of PHX’s extremely weak inhibition of CPT-1 it was “highly questionable that the effects are due to CPT1 inhibition”.[49] The chemical properties of PHX would also suggest relatively poor selectivity and specificity.[74] This is corroborated by the data in Table 1 which emphasizes the multiplicity of PHX targets and importantly, all available data point to PHX exerting its *least* potent effect on CPT-1 (Table 1). However, it is recognized that functional pleiotropy (i.e. drug promiscuity), which we have previously considered as ‘magic shotguns’ versus ‘magic bullets’[75], can be beneficial.[76] This is discussed this further in section 7.

In consideration of the other reported targets of PHX (Table 1), the relevance of competitive inhibition of CPT-1 by PHX *in situ* (characterized by an IC_{50} of 77 μM determined in isolated mitochondria membrane preparations[71]) may be questioned. Moreover, the inhibition of CPT-1 by malonyl-CoA is 10-100 times more potent (IC_{50} of 0.6 - 6.3 μM depending on the local concentration of palmitoyl-CoA (25 μM or 150 μM, respectively))[77] with a recent *ex vivo* study suggesting that malonyl-CoA produces a 33% inhibition of CPT-1 activity in resting (non-stimulated) muscle.[77] However, it may be important *in vivo* that the actions of PHX on CPT-1 are mechanistically different to those of malonyl co-A and oxfenicine (IC_{50} of CPT-1 inhibition = 11 μM[78]) and it has been suggested that PHX acts at a protected mitochondrial site not amenable to proteolysis i.e. in the plane of the membrane.[15, 71, 79] It is also intriguing that the specific counterion used in the preparation of the clinical formulation of a drug may have a profound impact on its physico-chemical properties, including logP.[80] This is especially pertinent for maleate which has been shown to directly influence cellular metabolism via promoting the utilization of glucose and the accumulation of fatty acids.[81, 82] The contribution of the maleate ‘counterion’ to the effects attributable to PHX, especially in the context of altered metabolism, warrants further investigation.[83]

Recent proteomic and metabolic studies have corroborated a complex effect of PHX on metabolism.[84, 85] In a study of mice treated for 4 weeks at steady-state PHX plasma

concentration, Yin and colleagues reported the activation of the pyruvate dehydrogenase complex (PDH) (Figure 1) and a "rebalancing of carbon and nucleotide phosphate fluxes, fuelled by increased lactate and amino acid uptake, to increase metabolic flexibility and maintain cardiac output".[84] Furthermore, Ceccarelli determined a greater potency of PHX on ketone body (KB) generation and beta-oxidation (FAO) inhibition in rat and human hepatocytes (IC_{50} = 14.8 and 22.4 μM, respectively) than on CPT-1 inhibition (IC_{50} > 100 μM).[49] Together these data advance the idea that the therapeutic effects of PHX may be due, in some part to *bona fide* metabolic modulation, but the notion of PHX being a potent inhibitor of CPT-1 needs further evaluation.

To this end, the effects of PHX on reduced ROS may be more attributable to direct inhibition of NADPH oxidase (NOX2) rather than effects on cardiac energetics *per se.*[86] Also, Unger and colleagues reported that in non-ischaemic working rat hearts, "perhexiline increases myocardial efficiency by a mechanism(s) that is largely or entirely independent of its effects on CPT".[87]

(5) PHX metabolism, physico-chemistry and tissue accumulation

A well-known consequence of longer term (unmonitored) PHX administration (typically > 3 months)[88] is lipidosis-induced hepato- and neuro-toxicity, hypoglycaemia and weight loss.[31, 34, 89-94] However, there is now a more complete understanding of the risk factors predisposing individuals to adverse effects, including patient-specific CYP2D6 status.[95-98] Oxidative metabolism of PHX produces mono- and di-hydroxylated metabolites. The predominant metabolite detected in plasma - *cis*-4 hydroxyperhexiline - is found at concentrations higher than PHX.[99, 100] Those individuals with CYP2D6 insufficiency ('poor metabolizers') have a reduced capacity to form this metabolite[101] and thus PHX is contraindicated in these individuals. This association between drug chirality and toxicity is also important[99, 100, 102] since there is reported stereoselectivity of (+) and (-) enantiomers to metabolism.[95, 100, 101] The asymmetry of the C2 of the piperidine ring means that PHX is administered as a racemic mixture of (+) and (-) enantiomers. The (+)-PHX enantiomer is

cleared more slowly from the plasma[103-105] and is associated with a more pronounced toxicity profile.[104, 106] Sallustio's recent patent on the use of (-)-PHX seeks to negate the issue of toxicity and other problems possibly linked to the complex mixture of different stereoisomers and metabolites of unknown efficacy arising from administration of the unresolved racemic mixture.[106, 107]

Awareness of the issues considered above, coupled with optimised dosing regimens and plasma drug monitoring to maintain a therapeutic range between 0.15 -0.6mg / L (approximately 0.5-2 μM)[108-110], have led to the re-introduction of PHX in the UK on a named patient basis. However, phospholipidosis-linked toxicity (similar to that observed with chronic amiodarone exposure)[111] raises the issue of accumulation in tissues which requires further consideration of PHX from a physico-chemical perspective.

PHX is very poorly water soluble (limit of solubility of 0.0608 mg / L; approximately 0.2 μM) and highly lipophilic- the logP, the octanol-water partition coefficient used as a measure of lipophilicity is 6.2, comparable to that of amiodarone, a drug for which membrane accumulation is a causal factor in its serious adverse effects (Table 2). Given the very low water solubility of PHX, at safe clinical dosing levels (i.e. plasma concentration of drug between 0.15 and 0.6 mg / L), the ratio of $[PHX]_{unbound}$ to $[PHX]_{bound}$ would be approximately 40:60% and 10:90%, respectively. Importantly then, since under normal (monitored) dosing conditions PHX would already be at its limit of solubility, any increase in the concentration of PHX upwards of reportedly 'toxic' plasma levels of drug (i.e. > 0.6 mg / L) would simply mean that more of the drug existed in a bound state in the plasma. The notion therefore that monitoring plasma levels of PHX does not give a true indication of drug distribution was recently confirmed by the remarkable finding of Drury and colleagues that the concentrations of PHX in human right atria and left ventricles were 6.0 and 10.0 mg / kg, respectively- approximately 100- to 170- times higher than the amount of PHX unbound ('free') in the plasma.[112] The massive accumulation of drug was corroborated by the same authors in a recent study.[103]

So how does PHX accumulate to such high levels in tissue? We describe the possible mechanisms that underpin accumulation of the drug in section 6, but it is first necessary to consider the impact that drug ionization state will have on the modes of transfer across cellular membranes. The pKa of PHX is 10.58 so 99.9% of the drug would exist in the charged (cationic) form in the plasma at pH7.4. As discussed above then under therapeutic dosing regimens (i.e. at its limit of solubility of 0.06 mg / L), effectively all of the drug would exist in the cationic form (≈ 99.9%, 0.05994 mg / L) with the neutral form present at just 0.00006 mg / L. If the tiny amount of the neutral form of the drug (≈ 60 ng / L in the plasma) was the only species able to transfer across the cell surface membrane, myocardial concentrations of the drug at 6 – 10 mg /kg [103, 112] would establish an intracellular:plasma concentration gradient of 100,000:1. It is inconceivable that such massive accumulation could be supported by simple passive transfer of the neutral form of the drug from the plasma into cells, even if one considers that the neutral species that does partition across the surface membrane would be immediately protonated (to its cationic form) in the cellular environment (Figure 2). This therefore raises the likelihood that other factors contribute to the cellular accumulation of PHX. First, the peculiar hydrophobicity properties of PHX may promote the integration of the cationic species into the membranes. Second, and probably the most likely contributor to the cellular accumulation of PHX, is that organic ion transporter proteins (TP) present in the surface membrane are likely involved in the active uptake of the cationic form of the drug into cells, as has been reported to occur with amiodarone, a drug with a very similar physico-chemical profile to PHX.[113]

(6) Possible mechanisms of PHX accumulation in tissues.

Some of the acute effects of PHX, at least in experimental systems, are rapidly reversible. In chick embryo ventricular cells, Barry *et al.* described the negative inotropic effect of PHX measured after a seven minute equilibration to reach steady state drug effects which was quickly reversed by washing out the drug.[114] However, as described in section 5, under

conditions of clinical dosing it appears that the therapeutic (and in some cases the hazardous) effects of PHX are primarily mediated by longer term accumulation inside cells.

Figure 2 describes some of the mechanisms that may contribute to PHX accumulation in the myocardium. Any cellular accumulation would be dependent on factors that disturb the free equilibrium between the cells and plasma and thus the only way that the drug can accumulate to levels grossly in excess of the plasma concentration is if there is an intracellular binding target or the relatively stable association of the drug with lipid membranes. Consequently, the scenario depicted in Figure 2A which describes the loading of cytoplasm with high $[PHX]_{free}$, and which may be commonly thought of as 'cytoplasmic accumulation', cannot occur. Also refuting the possibility that high cytoplasmic $[PHX]_{free}$ (as depicted in Figure 2A) occurs *in situ* is that under these conditions the surface membrane Na^+-, K^+- and Ca^{2+} channels would also be exposed to high concentrations of drug. Since the mode of channel inhibition is through drug interaction at the cytoplasmic face of the channel and in consideration of the comparatively low IC_{50} values for PHX-channel interaction (Table 1) these channels would be profoundly inhibited. This would result in catastrophic impact on heart rhythm and rate, effects that are conspicuously absent in PHX treated patients (although a heart rate lowering effect of PHX in other species has been reported[115, 116]).

Cellular accumulation could however feasibly occur via a mechanism that involves PHX binding to cytoplasmic moieties which would allow the drug to partition against its concentration gradient (Figure 2B). It is important to note though that in these circumstances, high levels of intracellular accumulation would not translate into high levels of drug concentration, and the levels of 'free' PHX in the cytoplasm would be low (Figure 2B). It is possible though that low levels of 'free' PHX in the cytoplasm, which would predominantly be in the cationic form (see above), would still be sufficient to modulate surface membrane ion channel activity (Table 1).

There is evidence of preferential association of PHX with phospholipid membranes, particularly those characterized by regional 'charge' gradients (e.g. H^+ gradients across

mitochondrial and lysosomal membranes)[59, 71, 117] which may contribute to intra-cellular localization of very high drug concentrations at these organelles. Such a mechanism could also conceivably lead to cellular 'trapping' of PHX. As to whether the protonation of PHX in the cytoplasm of the cell would lead to a buffering of H^+ ions and as a consequence modulate cellular pH, we believe this to be unlikely. In our view, any effect of PHX on pH normalization should remain focused on the relative balance of pyruvate-to-lactate which is dictated by the schemes outlined in Figure 1.

From its lipophilicity profile, the partitioning of PHX into membranes (especially mitochondrial membranes once it is protonated inside the cell) is likely to be rapid and it has been reported that high concentrations of drug applied extracellularly (25 μM) affected metabolism and promoted cellular toxicity within 24h.[89] Even more striking is the direct biochemical evidence for the decreased utilization of fatty acids (by approximately 35%) in rat hearts perfused with 2 μM PHX within a one-hour experimental window.[59] The timings of these effects point to either rapid cellular accumulation or alternatively, to an acute indirect modulation of metabolism (i.e. downstream from inhibition of surface-membrane ion channels).

There are two plausible scenarios in which longer-term accumulation of PHX could modulate CPT-1 and its other targets at the IC_{50}s described in the literature (Table 1). First, if CPT-1 and the other targets **are** the target for PHX binding (Figure 2C). Under these conditions one would expect marked inhibition of surface membrane ion channel fluxes and, for the reasons outlined above, this is unlikely to occur *in vivo*. Specifically with regards CPT-1 though, the scheme described in Figure 2C may seem unlikely because of the endogenous competition from the much more potent malonyl CoA and the very high IC_{50} of PHX (>100 μM in a *P. pastoris* expression system)[49] (Table 1). However, PHX interacts with CPT-1 via an atypical mechanism that possibly involves interaction within the plane of the membrane (see section 4, above) and thus could reach very high concentrations in the vicinity of CPT-1. Such massively localized accumulation of PHX at CPT-1 would constitute 'active-site concentration'[111] although little is known as to how this phenomena may

influence drug-target inhibition. The idea that PHX preferentially partitions in the extravascular pericardial compartment or pericardial fat (as occurs with amiodarone)[49] is not supported by Drury's study which determined very high levels of PHX accumulation in myocardial tissue obtained via biopsy of patients undergoing coronary artery bypass surgery which was presumably free of pericardial fat.[112]

The second scenario for chronic PHX accumulation, and in our view the most likely mechanism that reconciles the low potency of PHX for CPT-1 with its actions on this target and on other ion channels is shown in Figure 2D. In this scheme PHX distribution is heterogeneous and (1) it accumulates in the membrane, (2) it interacts directly with CPT-1 and (3) some of the drug is free in the cytoplasm as a result of an equilibrium set up between cytoplasmic binding factor(s) and the bulk hydrophilic environment. All of these distributions though are subject to saturability such that eventually all targets/binding partners would be PHX bound or associated with intracellular moieties. After dosing previously drug naïve patients at a plasma concentration of approximately 1 μM, concentrations of PHX in ventricular tissue reached over 10 mg / kg (approximately 36 μM) within 30 days of drug administration (median 8.5 days).[112] Moreover, although the PHX accumulation in these tissues was directly correlated with plasma concentration of drug and length of therapy there was **no** evidence of saturability of uptake over the time of study.[112] In a more recent follow-up, the conclusion of Chong and colleagues that PHX does exhibit saturability is not supported by their data (see[103], Fig 2A and B). Given these observations, it is difficult to conceive of how long-term PHX administration, even with maintenance of plasma concentrations of the drug at around 1 μM, would not lead to the steady accumulation of toxic levels of PHX. However, one of the authors of this article (ZY) has been treating some HF patients with PHX for over ten years; this chronic administration of PHX is well tolerated and the symptomatic improvements are substantial. Clearly, there are factors that affect PHX tolerance and accumulation that remain incompletely understood.

Finally, one commonly overlooked feature of CPT-1 is its widespread tissue distribution. CPT-1 is expressed at lower levels in the heart than in other tissues including

kidney, liver, pancreas, lung and intestine.[49] The issues considered above would presumably apply equally in these tissues and the reported patterns of hepato-, neuro- and gastrotoxicity are consistent with this idea.

(7) Expert Opinion.

In this article we have considered issues relating to the chemistry, mechanism of action, tissue accumulation and clinical benefits of PHX. An improved understanding of PHX drug safety and monitoring has underpinned its use in patients with HF and the evidence for its therapeutic efficacy in this context is strong. However, the disparate clinical endpoints following PHX administration in HF arising from distinct aetiologies (e.g. ischaemic- versus non-ischaemic HF) suggest that the mechanism of action of PHX is complex and not fully understood.

The more recent literature is dominated by the concept that the beneficial effects of PHX are due primarily to its inhibition of myocardial CPT-1. Contrary to this dogma, having reviewed all the available evidence, including key patents relating to the clinical usage of PHX and detailed studies of drug-target interactions, we suggest that it is very unlikely that the *major* effects of PHX are due to CPT-1 inhibition. Furthermore, over the last twenty years or so the preoccupation with CPT-1 as the most relevant target of PHX has overshadowed a substantive body of older, but very credible evidence, for PHX affecting the extra-cardiac circulatory system. It is entirely likely that the therapeutic benefits of PHX in HF are due to combined cardiac and vascular effects (e.g. vasodilation).

The robustness of some of the statements made regarding PHX and CPT-1 inhibition is at stark odds with the limited evidence supporting such a mechanism that can be found in the literature. In a recent and detailed analysis of CPT-1 inhibitor chemistry, Ceccarelli effectively debunked the idea that perhexiline is a physiologically relevant CPT-1 inhibitor.[49] However, we cannot completely exclude the possibility that **minor** effects of PHX on CPT-1 underpin disproportionately large effects on myocardial function, especially since there is massive accumulation of the drug in atrial and ventricular tissue. To reconcile these

arguments, it is plausible that the symptomatic improvements determined in HF patients are due to the fact that PHX is **not** a good CPT-1 inhibitor in an *in vivo* setting and that any clinical utility is dependent on only partial CPT-1 inhibition. Horowitz and colleagues, who first positioned PHX as an exemplar CPT-1 inhibitor[60, 71], have previously acknowledged this likelihood, albeit from the perspective of toxicity rather than efficacy- "the adverse reactions encountered with perhexiline and indeed those of other CPT-1 inhibitors (e.g. etomoxir) are the consequence of excessive CPT-1 and hence fatty acid metabolism inhibition".[71] Like PHX, the antianginal trimetazidine exerts multiple effects via multiple mechanisms (see e.g.[118]); it is a potent inhibitor of beta-oxidation of free fatty acids[119], but only a weak CPT1 inhibitor[120], and has been shown to reduce fibrosis[121] and to normalise aberrant Ca^{2+} handling in cardiomyocytes obtained from post-injured myocardium.[122] Moreover, the finding that etomoxir, the only drug considered as a *bona fide* CPT-1 inhibitor by Ceccarelli and colleagues[49] evoked severe hepatoxicity [123] would also suggest that appreciable inhibition of CPT-1 *in vivo* is not clinically useful. Thus although there is undeniable merit in developing new 'metabolic modulators', approaches that aim to develop selective CPT-1 targeted drugs with enhanced biological half-life and/or increased potency should be viewed with caution. It is also likely that targets other than CPT-1 present alternative strategies for regulating myocardial FA fluxes.[49, 124]

The linked nature of the composite systems involved in metabolic regulation (i.e. the complex synchronization of ion handling, metabolite sensing, substrate utilization, energy supply-and-demand) means that reconciling any effects on altered energy metabolism with the specific effects on discrete molecular targets is extremely difficult. With this in mind, the evidence that PHX effects are mediated by its actions on a multiple ion channels and other cellular components that impinge on aspects of cellular metabolism is more convincing (Table 1). In Figure 2D, we propose a scheme which considers the effects of PHX via 1) the inhibition of surface membrane ion channels via low levels of free drug in the cytoplasm and 2) accumulation in mitochondrial membranes which leads to a highly localized concentration of drug in the vicinity of CPT-1 resulting in its partial inhibition.

In summary, we suggest that due to its physico-chemical properties, PHX is a drug of low potency, low specificity and low selectivity. As a result, the pleiotropic actions of PHX on a diverse range of molecular targets, some of which remain to be properly defined, contribute to its therapeutic benefit in HF.

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Conflict of interest

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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* of interest

** of considerable interest

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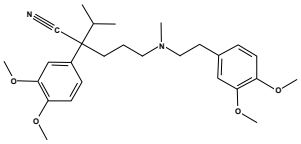
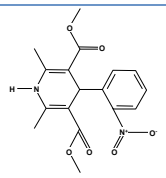
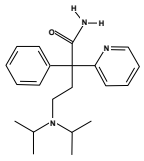
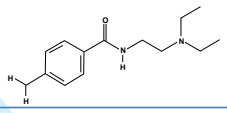
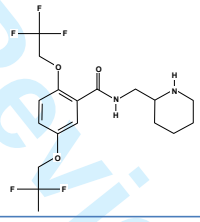
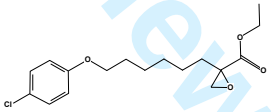
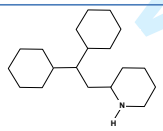
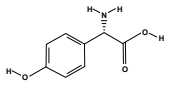
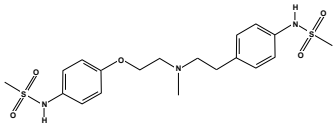
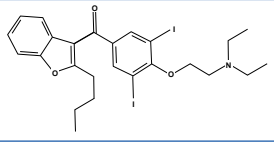
Rank order of potency	Target	IC_{50} (μM)	Experimental system	Ref.

Table 1. Rank order of PHX inhibitory potency.

1	$K_v1.5$ (IK_{ur})	2	Recombinant Kv1.5 in HEK cells	[125]
2	NADPH oxidase	2 - 6	Neutrophils, cardiac fibroblasts	[69, 86]
3	HERG	6 - 8	Recombinant HERG in CHO cells	[126, 127]
4	'Fast' Na^+ channels	10 - 30	Rat heart membrane / synaptosomes	[128]
5	Ca^{2+} channels	1 - 50	Rat heart membrane	[128]
6	NADH-CoQ (Complex I)	23 - 68	Rat heart mitochondria	[129]
7	Na^+/K^+-ATPase	50	Rat brain homogenate	[117]
8	CPT-2	73 - 79	Recombinant *Pichia pastoris* membrane / rat heart mitochondria	[49, 79]
9	CPT-1	77 - >100	Rat heart mitochondria / recombinant *Pichia pastoris* membrane	[49, 79]

$K_v1.5$, K^+ channel carrying the ultra-rapid delayed rectifier current (IK_{ur}); HEK, human embryonic kidney; CHO, Chinese hamster ovary; HERG, K^+ channel encoded by the human ether-a-go-go related gene that carries the rapid delayed rectifier current (IK_r).

Table 2. Chemical names, structures and logP values of Ca^{2+}-, Na^{+}-, K^{+}-channel and CPT inhibitors.

Drug	IUPAC name	Structure	logP	Primary target
Verapamil	(RS)-2-(3,4-dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino}-2-(propan-2-yl)pentanenitrile		3.8	L-type Ca^{2+} channel
Nifedipine	3,5-dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate		2.2	L-type Ca^{2+} channel
Disopyramide	(RS)-4-[bis(propan-2-yl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide		2.6	Na^{+} channels
Procainamide	4-amino-*N*-[2-(diethylamino)ethyl]benzamide		0.9	Na^{+} channels
Flecainide	(RS)-*N*-(piperidin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide		3.8	Na^{+} channels
Etomoxir	ethyl 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate		4.5	CPT
Perhexiline	2-(2,2-dicyclohexylethyl)piperidine		6.2	See Table 1
Oxfenicine	(2S)-2-amino-2-(4-hydroxyphenyl)acetic acid		0.2	CPT?
Dofetilide	*N*-[4-(2-{[2-(4-methanesulfonamidophenyl)ethyl](methyl)amino}ethoxy)phenyl]methanesulfonamide		2.1	K^{+} channels
Amiodarone	{2-[4-(2-butyl-1-benzofuran-3-carbonyl)-2,-6-diidophenoxy]ethyl}diethylamine		7.6	K^{+} channels

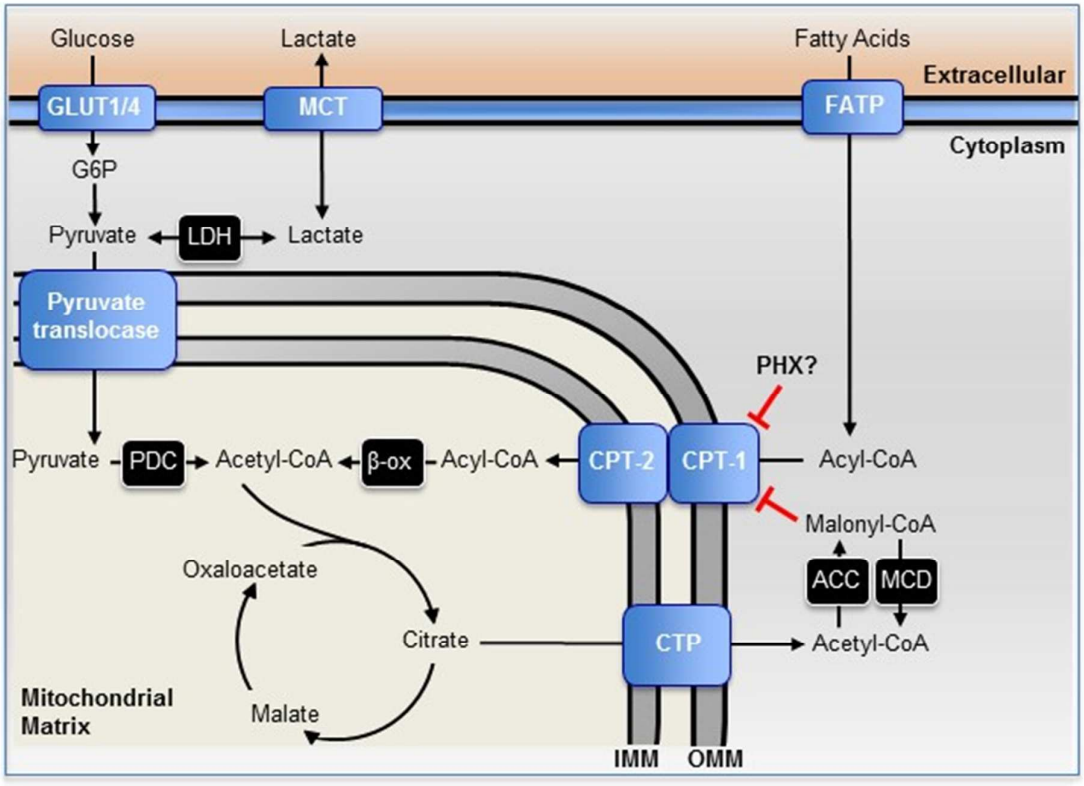


Figure 1. Endogenous inhibition of CPT-1 by malonyl-CoA

Studies using ^{13}C-radiolabelled etomoxir as a probe for CPT distribution revealed that in the heart, CPT-1 is expressed to higher levels than CPT2.[50] This raises the possibility that not all CPT1 is 'paired' with CPT2 as is depicted here. CPT-1B is the predominant cardiac isoform and is more sensitive to malonyl co-A inhibition than CPT-1A (IC_{50} of 0.03μM vs. 0.3-1μM, respectively)[49].

GLUT, glucose transporter; G6P, glucose-6-phosphate; LDH, lactose dehydrogenase; FATP, fatty acid transporter protein; MCT, monocarboxylase transporter; PDC, pyruvate dehydrogenase complex, β-ox, β-oxidation; CPT-1, carnitine palmitoyltransferase 1; CPT-2, carnitine palmitoyltransferase 2; CTP, citrate transporter protein; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane.

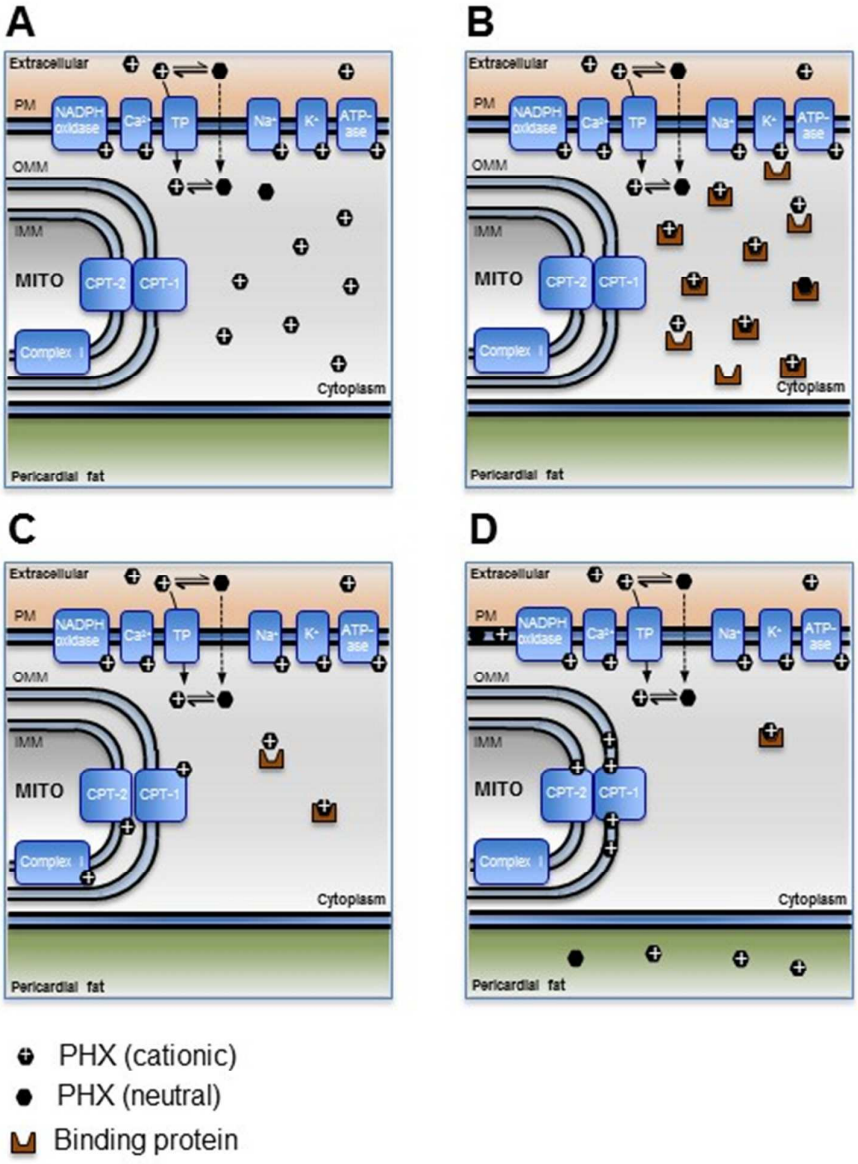


Figure 2. Modes of cellular accumulation of PHX

At pH7.4 approximately 99.9% of PHX would exist in the cationic form in equilibrium with approximately 0.1% as the neutral species. As described in section 5, organic ion transporter proteins (TP) probably play an important role in the active uptake and intracellular accumulation of PHX, as has been described for amiodarone.[113]

(A) Increased cytoplasmic [PHX] and high intracellular concentrations of 'free' (unbound) PHX would not be possible under equilibrium conditions if plasma [PHX] was maintained at much lower levels.

(B) Cellular accumulation of PHX could occur if PHX bound with reasonably high affinity to a cytoplasmic target / binding partner.

(C) Selective accumulation of PHX by direct binding to its reported targets (see Table 1).

(D) Heterogeneous distribution of PHX via partitioning of neutral and cationic species of drug into mitochondrial membranes which promotes high localized concentrations around CPT-1. Some [PHX] in the cytoplasm available to interact with surface membrane ion channels and, over time, accumulate in pericardial fat.